Chapter 3

recA mediated spontaneous deletion of the *icaADBC* operon in clinical *Staphylococcus epidermidis* isolates; a new mechanism of phenotypic variations

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Abstract

Phenotypic variation of *Staphylococcus epidermidis* involving the slime related *ica*-operon results in heterogeneity in surface characteristics of individual bacteria in axenic cultures. Previously, we found that in clinical *S. epidermidis* isolates, loss of *ica* was irreversible and independent of the mobile element IS256. Therefore we investigated the role of LexA and RecA in the observed irreversible switching from *ica*-positive to *ica*-negative in clinical *S. epidermidis* isolates. In high frequency *S. epidermidis* switching strains, spontaneous mutations in *lexA* were found which resulted in deregulation of *recA* expression, as shown by real time PCR. RecA is involved in genetic deletions and rearrangements and we postulate a model representing a new mechanism of phenotypic variation in clinical isolates of *S. epidermidis.* This is the first report of *S. epidermidis* strains irreversibly switching from *ica*-positive to *ica*-negative phenotype by spontaneous deletion of *icaADBC*, which represents a new mechanism of phenotypic variation.

Introduction

Staphylococcus epidermidis is recognized with increasing frequency as an opportunistic and nosocomial pathogen, particularly in association with infections related to indwelling medical devices. Many studies have demonstrated that slime production, as governed by genes within the *icaADBC* operon, is one of the most important virulence factors in the pathogenicity of *S. epidermidis*[1,18]. Christensen *et al.*[4] showed that slime production by *S. epidermidis* is not a stable phenomenon but undergoes phase variation. Recently, insertion or excision of transposon IS256 has been identified as one of the mechanisms responsible for regulation of *ica*-expression, either in the structural operon[18] or other genes encoding proteins that regulate *ica*-expression such as *rsbU* and *sarA*[5]. It is a reversible mechanism since the *ica*-positive phenotype can be resumed from the *ica*-negative phenotype after repeated sub-culturing. However, Arciola *et al.*[2,3] have reported that a significant proportion (42%) of clinical *S. epidermidis* isolates are *ica*-negative and IS256 was never found inside the *ica* locus. They concluded therefore that this transposon is probably not a natural occurring mechanism for on/off switching of biofilm production.

We previously identified irreversible, genotypic, switching from a *ica*-positive to a *ica*-negative phenotype in several clinical *S. epidermidis* isolates. After extended incubation on Congo Red Agar (CRA) of the *ica*-positive (black) and *ica*-negative (red) colony and repeated sub-culturing, no black colonies could be regained from red colony inocula. The black colonies were consistently smaller than the red colonies, while there was extensive flocculation and reduced turbidity in broth cultures inoculated with black colonies. Importantly, the red variants from the five strains studied had permanently lost *icaA* and *icaC* from their genome. This switching phenomenon was independent of IS256 and irreversible in nature[15].

The majority of biomaterial-related isolates are *ica*-negative[3] and recently it was observed that the presence of the *icaADBC* operon represents a disadvantage when *S. epidermidis* colonizes the skin[16]. Therewith on one hand, *icaADBC*

represents a growth disadvantage while on the other hand, the ability to express different slime-producing phenotypes could provide the staphylococci with a greater degree of flexibility for colonizing a range of biomaterials[4,12]. The irreversible loss of the *icaADBC* operon could represent an important clinical phenomenon, and investigation of the mechanisms involved is desired.

A recent report on the genomic sequence of *S. epidermidis* ATCC12228 showed the complete deletion of the *icaADBC* operon and extensive genomic rearrangements of the region where the operon is normally found, as e.g. in *S. epidermidis* RP62A[17]. Similar as in *S. epidermidis*, high frequency of mutations is observed in *Yersinia pestis* that also involves black-red switching on CRA plates[8]. One of the mechanisms proposed involves recombinase A (RecA), catalyzing the genetic recombination between homologous regions of DNA. This enzyme is involved in the so-called SOS response; in the presence of damaged DNA, the expression of a number of genes involved in DNA repair is induced representing an inducible DNA repair and damage tolerance system. RecA acts to catalyse the recombination between homologous DNA, while LexA, a small (22 kDa) protein functions as a repressor at many operons of the SOS response, including both *recA* and *lexA* itself[7,9]. Interestingly, *S. epidermidis* ATCC12228, which contains extensive genomic rearrangements, contains a frame-shift mutation in *lexA*[17] resulting in a truncated protein.

The aim of the current study was to investigate the role of LexA and RecA in the observed irreversible switching from *ica*-positive to *ica*-negative in clinical isolates of *S. epidermidis.* We hypothesize, based on observations in *Y. pestis* and the observed mutation in *lexA* in *S. epidermidis* ATCC12228, that random mutations in *lexA* result in dysfunctional regulation of *recA* expression and consequently increased occurrence of genetic rearrangements and deletions (Fig. 1).

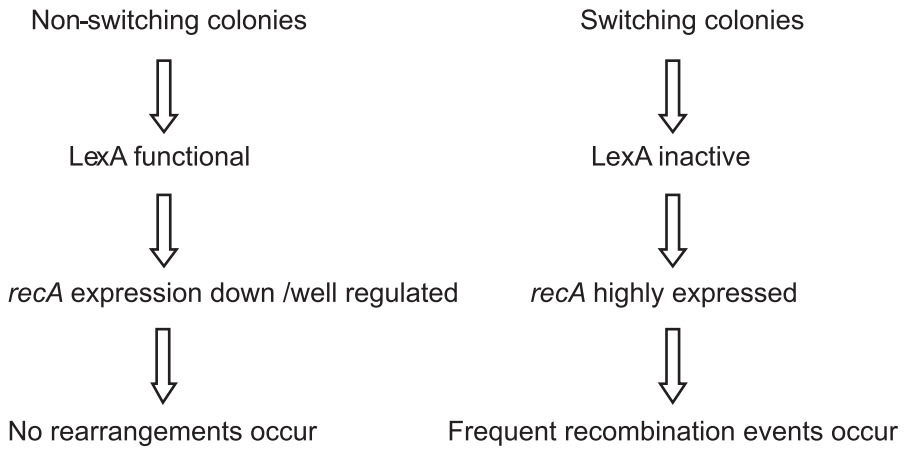

Figure 1. Hypotheses indicating the role of *recA* and *lexA* in the occurrence of spontaneous genotypic switching.

Materials and Methods

Strains and growth conditions

S. epidermidis strains (listed in Table 1) were grown in tryptone soya broth (TSB, Oxoid, Basingstoke, UK), as described previously (see chapter 2). The isolates were stored at -80°C in glycerol. *S. epidermidis* ATCC12228 and RP62A (ATCC35984) were used as reference strains from the literature.

Table 1. *S. epidermidis* strains used in this study showing phenotypic variation, i.e. black (*ica*-positive) and red (*ica*-negative) colonies on CRA plates and percentage switched cells observed on these plates[15]. Strain 46 is a black (*ica*-positive), non-switching strain used as the control strain.

S. epidermidis	*ica* status	Switched cells (%)
RP62A	+	0
ATCC12228	-	0
46	+	0
45	Mixed	39 ± 5
196	Mixed	93 ± 5
493	Mixed	15 ± 1
906	Mixed	79 ± 2
1098	Mixed	93 ± 11

Pulsed Field Gel Electrophoresis (PFGE)

To exclude the clonal relatedness of clinical isolated staphylococci, PFGE was performed as previously described[13,14]. Briefly, 0.5 mL of an overnight bacterial culture grown statically in TSB at 37°C was mixed with 2 µL of lysostaphin (Sigma-Aldrich, Steinheim, Germany) and 300 µL of low melting temperature agarose 2% (w/v). The resulting agarose blocks were incubated for 3 h at 25°C in 120 µL of restriction buffer containing 40 U of *SmaI*. Electrophoresis was performed in an agarose 2% (w/v) gel in a CHEF DR III apparatus (Bio-Rad, Hercules, CA, USA), with a constant voltage of 200 V and a pulse time increasing from 5 to 40 s over 20 h. The gels were stained with ethidium bromide and photographed.

DNA isolation, cloning, sequencing, and analysis of lexA genes

DNA isolation was performed as described previously in chapter 2. Briefly, bacterial cells from overnight cultures grown in TSB, were pelleted by centrifugation,

resuspended in 75 µL of demineralised water and treated with 25 µL of 1 mg mL^{-1} lysostaphin for 10 min at 37°C. Ten µL 1 mg mL^{-1} proteinase K (Sigma-Aldrich, Steinheim, Germany) in demineralised water and 200 µL of 0.1 M Tris-HCl (pH 7.5) was added and incubated for 30 min at 37°C. Samples were heated to 100°C for 5 min after which cell debris and unlysed cells were removed by centrifugation (21,000 x *g* for 10 min). The DNA in the supernatant was ethanol precipitated, dissolved in 100 µL 10 mM Tris-HCl, 1 mM EDTA (TE) and the concentration of DNA was determined using a Nanodrop UV/VIS spectrophotometer (Isogen-Biosolutions Inc., Maarsen, the Netherlands). Samples were stored at -20°C until needed.

Furthermore, *lexA* was amplified from the isolated genomic DNA using the primers listed in Table 2a. PCR products were then cloned into pCR2.1 (TA-cloning kit, Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. The presence of inserts was confirmed by restriction analysis using EcoRI. Positive clones were used for transformation of *Escherichia* coli DH5α and plates containing positive clones were sent for commercial sequencing (Baseclear, Leiden, The Netherlands).

The *lexA* sequence of *S. epidermidis* isolates was analyzed using Basic Local Alignment Search Tool assessed from *http://www.ncbi.nlm.nih.gov/blast* and compared with the sequence of LexA translated protein from *S. epidermidis* RP62A published in the Gen Bank.

Table 2a. Primer sequences for PCR. bp = base pair.

Primer	Sequence (5'-3')	Product size (bp)
lexA forward	GAACTCACTAAGCGACAAA	592
lexA reverse	ATTACTTTACCTACTACAATGACA	

Table 2b. Primers and their sequences for real time PCR. AT = optimal annealing temperature.

Primer	Sequence (5'-3')	Product size (bp)	AT (°C)
gyrB-3 forward	GGAGGTAAATTCGGAGGT	129	57.1
gyrB-3 reverse	CTTGATGATAAATCGTGCCA		
recA-1 forward	AAAGTTCAGGTAAGACGACAG	277	54
recA-1 reverse	TCCCATTTCACCTTCAATTTCAG		

Total RNA isolation and real time PCR analysis of recA expression

Total RNA was isolated from 24 h cultures grown in TSB at 37°C. Cells were harvested by centrifugation, and frozen at -80°C. Samples were thawed slowly on ice and resuspended in 100 µL water after which the bacterial suspension was frozen in liquid nitrogen. Frozen bacteria were grounded using a mortar and pestle. Total mRNA was isolated using the Invisorb® Spin Cell RNA Mini Kit according to the manufacturer's instructions (Invitek, Freiburg, Germany). DNA was removed using the RNeasy mRNA clean-up protocol (Qiagen, Valencia, CA, USA) and absence of genomic DNA was checked by PCR prior to reverse transcription. For all samples 35 cycles of PCR using the *gyrB* primer set (Table 2b) did not result in any detectable signal. One µg of total RNA was used for cDNA synthesis (Iscript, Bio-rad, Veenendaal, The Netherlands) according to the manufacturer's instructions. Reactions were prepared using the CAS-1200™ pipetting robot (Corbett Life Science, Sydney, Australia). The real time PCR used the following program: 95°C for 3 min, 40 cycles of 95°C for 15 s, primer specific annealing temperature (Table

2b) for 15 s, elongation at 72°C for 15 s. Expression levels of *recA* (see primers in Table 2b), were calculated using the $2^{-\Delta\Delta C_T}$ method[11] using *gyrB* as a reference gene and *S. epidermidis* RP62A as the reference strain.

Results

PFGE

In Fig. 2 the PFGE of the switching and reference strains are presented. The patterns showed different bands for the strains isolated from a black (*ica*-positive) and red colony (*ica*-negative), meaning that they were clonally unrelated. From the switching isolates, strain 493 and 906 showed different patterns of PFGE between black and red colonies. This indicated that in these two strains the number of recombination events is significant, there are many genetic rearrangements meaning that new strains develop. The other strains tested showed identical patterns of PFGE between black and red colonies resulting in derivatives from the same strain, probably illustrating a lower number of recombination events in those strains.

Cloning and sequencing of lexA

Upon sequencing of *lexA* of *S. epidermidis* ATCC12228 in our laboratory, no frame shift mutation was found, in contrast to a previous report, in which *lexA* appeared to contain a frame shift mutation resulting in a truncated protein[17]. Instead, multiple mutations resulting in two amino acid substitutions were observed (Fig. 3), both of which were within the essential N-terminal part of the protein[10]. From the five switching clinical isolates, only strain 196 contained amino acid substitutions at different positions within LexA (Fig. 3). The other four strains (not shown) contained mutations in *lexA*, but none resulted in apparent amino acid substitutions and showed the same sequence as RP62A. Sequencing of strain 46 (black control)

showed a conserved amino acid change (lysine for arginine) that probably does not affect LexA activity.

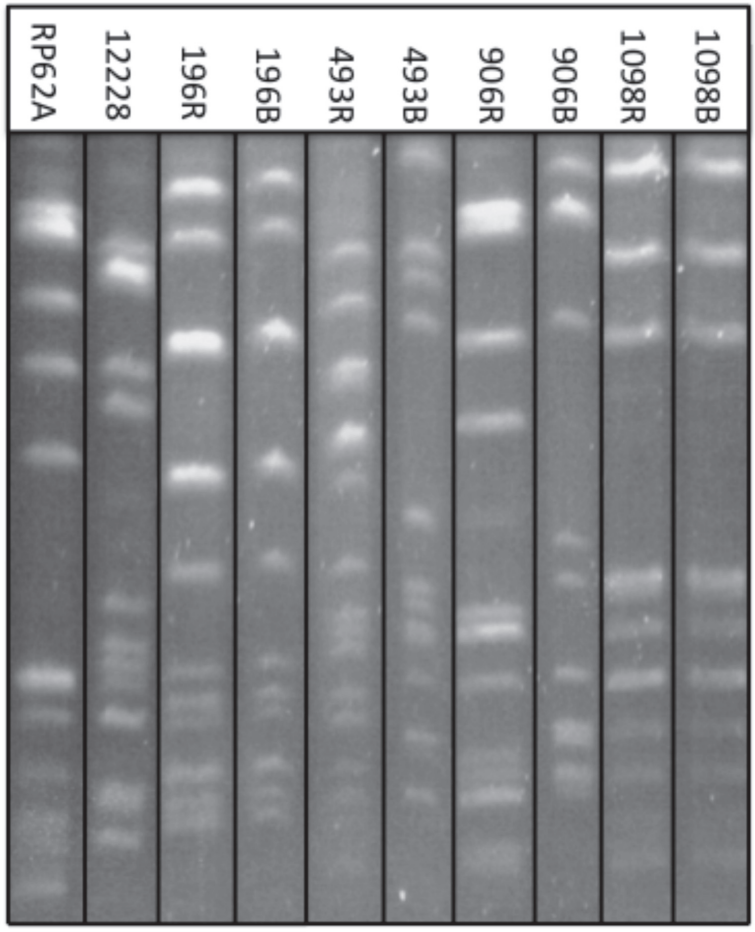

Figure 2. PFGE results of the *S. epidermidis* switching strains and the two non-switching reference strains RP62A and ATCC12228. For each strain the left lanes represent DNA isolated from a red (R) colony and the right lanes DNA isolated from a black (B) colony.

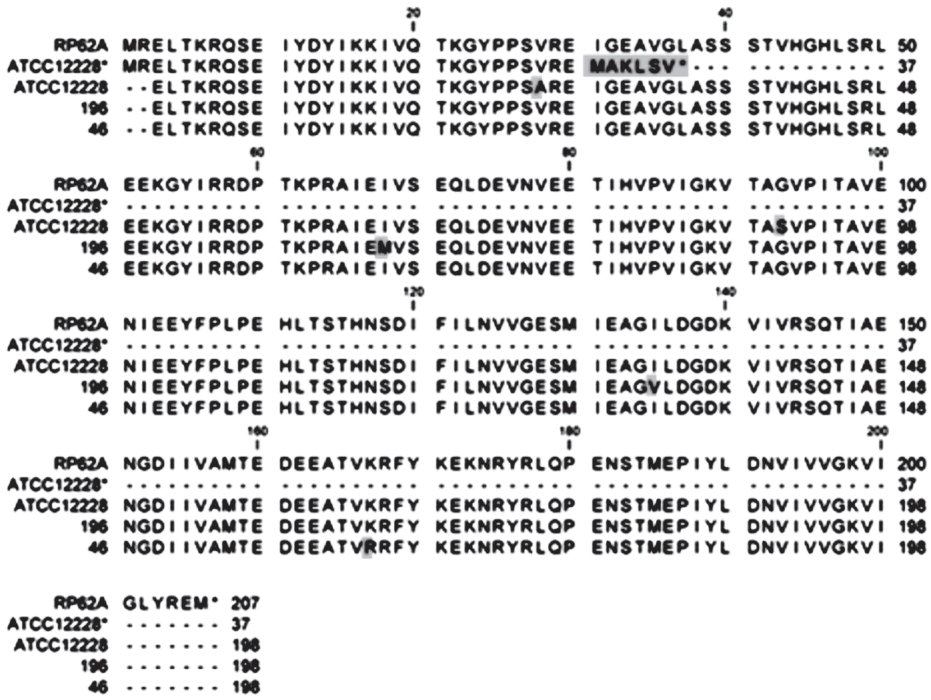

Figure 3. Sequence alignment of translated *lexA* sequencing products. The sequences are compared to the published LexA sequence of *S. epidermidis* RP62A. ATCC12228* indicates the truncated LexA sequence as published in the database[17], ATCC12228 is measured in this study. Mutated residues are indicated by gray shading.

Expression analysis of recA

The effect of the mutations in *lexA*, as observed in *S. epidermidis* ATCC12228 and one of the clinical isolates, on *recA* expression was analysed using real time PCR. Relative to the reference strain, *S. epidermidis* RP62A, *recA* was over-expressed 4 times in *S. epidermidis* ATCC12228 and 196, and 12 times in strain 906 (Fig. 4),

whereas the other three strains had no detectable expression of *recA* (data not shown).

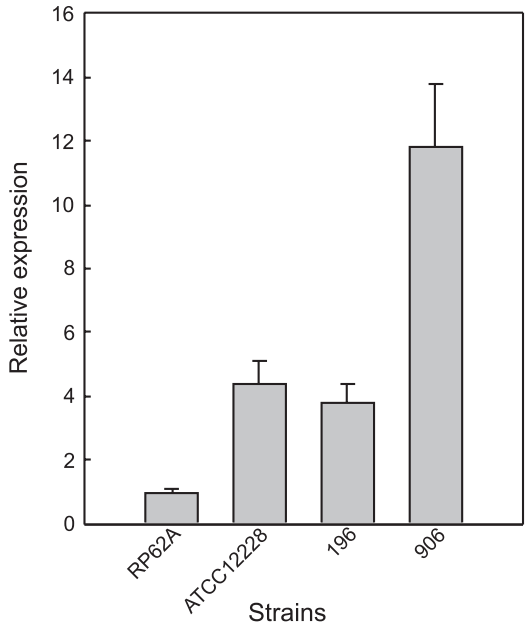

Figure 4. Relative expression of *recA* plotted using RP62A as a reference strain and *gyrB* as the reference gene. The $2^{-\Delta\Delta C_T}$ was calculated from the average C_T values.

Discussion

The five clinical *S. epidermidis* isolates used in this study showed phenotypic variations, determined from its biofilm forming ability, *ica*-status and electrophoretic mobility[15]. None of the isolates analysed originated from hospital acquired infection, but all were derived from patients that came into the clinic with infections thus limiting the chances of these isolates to be clonally related, as confirmed by PFGE assay (Fig. 2).

Previous studies have shown that absence of *ica* is common among clinical *S. epidermidis* isolates[3] and that in most of these *ica*-negative strains, IS256 is not present[2]. The fact that clinical isolates rapidly lose *ica* might explain that strains isolated from biofilms are *ica*-negative once in the laboratory environment[15].

The involvement of RecA, which functioned as a catalyst in DNA recombination events[7], in phenotypic variation of *Y. pestis* on CRA plates has been reported previously[8]. In addition, its activity is tightly regulated since inappropriate recombination can have serious consequences, such as deletion of genomic regions between repeats of genomic sequences[6]. In line with observations in *Y. pestis*, a high frequency of mutation is observed in several clinical *S. epidermidis* isolates used in this study. *S. epidermidis* ATCC12228, commonly used as an *ica*-negative reference strain, contains many inversions and rearrangements in its genome[17]. We hypothesized that the high frequency of deletion events is a consequence of mutations within *lexA* resulting in deregulation of *recA* expression and therewith a high frequency of recombination, insertion and genetic rearrangement events, causing a deletion of the *icaADBC* operon. Therefore *recA* expression was analysed in strains that had lost the *icaADBC* operon and appeared indeed upregulated in the strains ATCC12228, 196 and 906, as compared to RP62A (Fig. 4). Remarkably, strains 196 and 906 are the clinical isolates with high % of switched colonies on CRA plates (see Table 1). These data give experimental evidence that our hypothesis was responsible for the occurrence of spontaneous genotypic switching in about two out of five *S. epidermidis* strains included in this study.

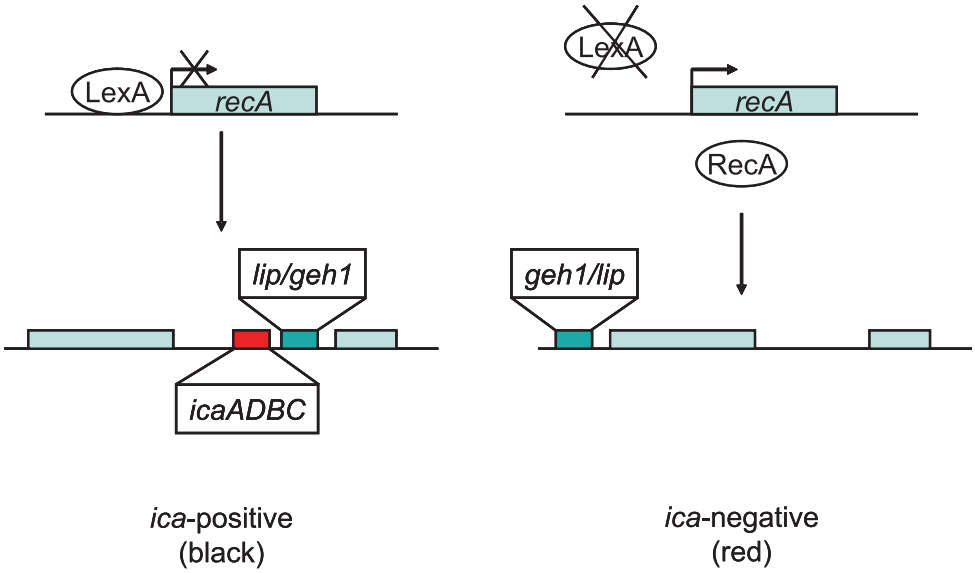

Figure 5. Schematic overview of the proposed series of events, leading to the deletion of the *ica*-operon. In the presence of LexA, transcription of *recA* is prevented, there are no genomic rearrangements and the *icaADBC* region is present thus resulting in black colonies on CRA plates. Due to mutations, LexA can no longer bind to the promoter region of *recA* resulting in RecA transcription and activity and the occurrence of genetic rearrangements and deletions. As a result the geh1/lip genes (and other regions) are inverted and the *icaADBC* region disappears thus resulting in red colonies on CRA plates.

For *Y. pestis* it was proposed that the high frequency of mutations could occur because of the lack of selection for congo red binding[8]. In addition, the ability to produce polysaccharide intercellular adhesin (i.e., carrying the *icaADBC* locus) confers for *S. epidermidis* a selective benefit in certain niches[16]. Because of the observed growth advantages of *ica*-negative *S. epidermidis* strains, it can be that there is a positive selection for *ica*-negative strains to develop due to increased fitness. Therefore we propose the following model for the observed switching (Fig. 5). Due to growth limitation of strains showing high expression of the *ica*-operon, selective pressure exists for mutants that lack this operon. These mutants develop

due to deregulation of *recA* as a consequence of (probably spontaneous) mutations in *lexA*. As a result of this mechanism, high frequency deletion of the *ica*-operon occurs. From the 105 strains isolated from blood, cerebrospinal fluid, pus and urine we used in Chapter 2, biofilm-negative phenotype was found in 85% of the strains tested. Additionally, Arciola and coworkers[3] surveyed the presence of *ica* in 400 clinical *S. epidermidis* isolates from prosthesis-associated (i.e. biofilm related) infections using a PCR method and found that 42% of the isolates were *ica*-negative[3]. If this random event results in deletion of the *icaADBC* operon this represents a growth advantage in broth (see chapter 2) and this would explain why the irreversible genotypic switching is commonly observed in clinical isolates, once they are cultured under ideal laboratory conditions[2]. The new switching mechanism described in the current study could explain the high occurrence of *ica*-negative clinical isolates as observed previously.

In conclusion, we observed a new mechanism of phenotypic variation in clinical *S. epidermidis* isolates, that deletion of *ica* appears to be caused by spontaneous mutations in *lexA*, which results in deregulation of *recA* expression leading to genetic rearrangements and deletions.

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